

TITLE OF THE INVENTION

METHODS OF IDENTIFYING AND DETERMINING THE EFFECTIVENESS OF
THERAPEUTIC PROCESSES OR AGENTS FOR THE DIAGNOSIS AND
TREATMENT OF SCHIZOPHRENIA AND RELATED DISORDERS

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a divisional of U.S. Application No.
08/833,703 filed April 8, 1997, entitled METHODS AND
MATERIALS FOR THE DIAGNOSIS AND TREATMENT OF SCHIZOPHRENIA
AND RELATED DISORDERS; the whole of which is hereby
incorporated by reference herein.

BACKGROUND OF THE INVENTION

Schizophrenia is a devastating neuropsychiatric disorder
which affects approximately 1% of the population and results
in serious disruption in the lives of afflicted individuals
and their families. Common symptoms include delusions,
conceptual disorganizations and visual or auditory
hallucinations, as well as changes in affective behavior. A
number of scales for the rating of symptoms and methods for
ascertaining the diagnosis have been developed, including the
DSM classification by the American Psychiatric Association
(Diagnostic and Statistical Manual of Mental Disorders (4th
edition), pp. 273-316, 1994), which have attempted to refine
the accuracy of clinical diagnosis. However, it is likely
that similar symptoms can result from several underlying
abnormalities, and diagnosis relying solely on clinical
symptoms is difficult and controversial, as well as
subjective, time-consuming and costly.

The cause or causes of schizophrenia remain obscure. A
defect in dopamine pathways of synaptic neuronal function is
a central feature of the most widely held etiopathogenic

theory (known as the Dopamine Hypothesis), with recent emphasis on the role of D₄-type dopamine receptors (Taubes, Science 265:1034-1035, 1994). However, studies to date have failed to identify abnormalities in the basic receptor structure, suggesting that dysfunction may result from an alteration in the dynamic regulation of receptor activity.

Dopamine receptors are members of a large superfamily of G protein-coupled receptors which share a high degree of structural similarity while recognizing a widely divergent array of substances which affect cellular function. Recent advances in the study of these receptors, including the development of 3-dimensional structural models (Teeter et al., J. Med. Chem. 37:2874-2888, 1994), have led to the identification of key locations on the receptors which can modulate their function and which therefore may be sites of malfunction in schizophrenia (Samama et al., J. Biol. Chem. 268:4625-4636, 1993). One such critical location or "hot spot" in the dopamine D₄ receptor is a methionine amino acid residue (Van Tol et al., Nature 350:610-614, 1991).

A number of clinical and metabolic studies have documented altered methionine metabolism in individuals with schizophrenia (Kelsoe et al., J. Neurosci. Res. 8:99-103, 1982; Ismail et al., Biol. Psych. 13:649-660, 1978; Sargent et al., Biol. Psych. 32:1078-1090, 1992). For example, the administration of methionine has been shown to elicit an acute psychotic reaction in persons with schizophrenia but lacks such an effect in normal individuals (Cohen et al., Biol. Psych. 8:209-225, 1974). Conversely, administration of S-adenosylmethionine has been shown to have antidepressant benefits (Kemali et al., Biochemical and Pharmacological Roles of Adenosylmethionine and the Central Nervous System, Pergamon Press, pp. 141-147, 1979).

Recently, these findings have been integrated upon the discovery that methionine residue #313 (human D₄ receptor numbering) of the dopamine D₄ receptor appears to be

abnormally modified in schizophrenic individuals (Deth, "Compositions and Methods for Detection of Schizophrenia," WO 96/37780, the whole of which is hereby incorporated by reference herein). Methionine residue #313 is normally modified by the addition of an adenosyl group to its sulfur atom via the action of a methionine adenosyltransferase (MAT) enzyme; however, individuals with schizophrenia are known to be deficient in MAT activity (Carl et al., Biol. Psych. 13:773-776, 1978) and, thus, are expected to possess a lesser amount of the modified form of the dopamine D₄ receptor. This deficiency is evident in a variety of tissues, including brain tissue and blood cells, particularly white blood cells, and is central to the biochemical diagnosis of schizophrenia.

BRIEF SUMMARY OF THE INVENTION

This invention is based upon the discovery that a biochemical abnormality in phospholipid methylation associated with schizophrenia is linked to the abnormal modification of the dopamine D₄ receptor and the clinical manifestations of altered dopamine neurotransmission. This discovery provides novel and empirical approaches for the diagnosis and treatment of schizophrenia and other related disorders.

This invention pertains to novel biochemical methods for assisting in the diagnosis of schizophrenia and other neuropsychiatric disorders, including, but not limited to, schizo-affective disorders, depression and dementias. The method of the present invention is based on the fact that dopamine receptor function is abnormal in individuals with schizophrenia, and upon the discoveries, described herein, that the dopamine D₄ receptor is a direct participant in the methylation of membrane phospholipids and that phospholipid methylation is abnormally altered in schizophrenic individuals. Consequently, an altered membrane fluidity may

play a significant role in producing the symptoms of schizophrenia.

5 In one embodiment of the present invention, the level of phospholipid methylation in a tissue sample, e.g., peripheral blood cells (such as lymphocytes), from an individual to be tested is measured, preferably using [¹⁴C]formic acid labelling or any similar method of labelling the methylfolate pool. Additionally, the level of contribution of the D₄ receptor to phospholipid methylation in the same sample is also determined. The measured levels are then compared with corresponding levels of the same indicators from a tissue sample of a normal individual; a lower level of phospholipid methylation activity in the tested individual compared with the normal individual is indicative of schizophrenia or a related neuropsychiatric disorder in the tested individual.

15 A lower level of D₄ receptor contribution to phospholipid methylation activity in the tested individual compared with the normal individual is indicative of schizophrenia specifically in the tested individual. Alternatively, a [sup>3]H]-methyl-methionine based method of phospholipid methylation activity determination is used, a more indirect assay.

20 This invention also pertains to novel methods for identifying therapeutic processes or agents for treatment of schizophrenia or related neuropsychiatric disorders using cultured cell lines transfected with the D₄ receptor gene. Processes or agents identified by the methods described herein can increase the amount of phospholipid methylation to the normal level.

25 Furthermore, this invention also pertains to novel methods for determining the effectiveness of therapeutic processes or agents for treatment of neuropsychiatric disorders, and in particular schizophrenia. In one embodiment of the invention, the effectiveness of a therapeutic process or agent can be assessed by making an

initial determination of the level of phospholipid methylation in a lymphocyte sample from an individual to be tested, administering the process or agent to be assessed, and making a subsequent determination of the level of phospholipid methylation in the lymphocyte sample from the individual. The corresponding levels of phospholipid methylation can be compared; an increase in the level of phospholipid methylation (preferably a normalization of methylation levels) indicates that the process or agent is effective for treating schizophrenia.

In a particular embodiment, the determination of the levels of phospholipid methylation is carried out using [¹⁴C] formic acid labelling of the methylfolate pool. Thus, the present invention has utility for the identification of agents and processes for use in the treatment of schizophrenia, and such agents, processes and drugs are also the subject of this invention.

Any tissues which display the schizophrenia-associated alterations in methionine metabolism are suitable for use in the methods of the present invention. Such tissues include brain tissue and red and white blood cells. Peripheral blood cells (especially lymphocytes) are particularly useful in the present invention because of their accessibility, allowing the methods of the present invention to be carried out on a blood sample from the individual, and because lymphocyte membranes contain the D₄receptor.

BRIEF DESCRIPTION OF THE DRAWINGS

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof and from the claims, taken in conjunction with the accompanying drawings, in which:

Fig. 1 is a prior art rendering of the dopamine D₄ receptor structure, shown the amino acid sequence of the

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human dopamine D₄ receptor and its proposed seven transmembrane helical elements (Deth, WO 96/37780). The cell membrane is shown as a rectangle with the extracellular surface at the top and intracellular surface at the bottom.

5 Methionine #313 (the "hot spot") is indicated with an arrow;

Fig. 2 is a schematic outline of metabolic pathways showing the use of [¹⁴C] formic acid or [³H] methionine to measure D₄ receptor-dependent and receptor-independent phospholipid methylation;

10 Figs. 3a, 3b and 3c show involvement of D₄ dopamine receptor in phospholipid methylation; and

Figs. 4a and 4b show phospholipid methylation in lymphocytes from persons with schizophrenia vs. normal controls.

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DETAILED DESCRIPTION OF THE INVENTION

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The "Dopamine Hypothesis" is the most widely held biochemical explanation for the etiology of schizophrenia and theorizes a defect in dopamine pathways of synaptic neuronal function, with recent emphasis on the role of D₄-type dopamine receptors. Dopamine receptors are members of the superfamily of G protein-coupled receptors. All G protein-coupled receptors share the basic structural motif of seven transmembrane-spanning helices, formed as the single polypeptide chain traverses the plasma membrane (O'Dowd, J. Neurochem 60:804-816, 1993). In the case of the dopamine receptor, the neurotransmitter dopamine diffuses into the central core of the receptor on the outer surface of the cell where critical amino acid residues provide specific recognition. The binding of dopamine and its recognition by the receptor cause an alteration in the conformation of the receptor, and this "active" conformation conveys the neurotransmitter signal to GTP-binding G proteins located on the inner surface of the cell membrane.

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Five subtypes of dopamine receptors have been identified, designated as D₁, D₂, D₃, D₄ and D₅. Based upon functional and structural similarities, the D₁ and D₅ receptors form a D₁-like receptor group, and the D₂, D₃ and D₄ receptors comprise a D₂-like group. Among dopamine receptors, the D₁-like receptors primarily complex with and activate the G protein G_s, while the D₂-like receptors activate the G_i and/or G_o proteins. The potency of neuroleptic drugs in treating schizophrenia has been found to be closely correlated with their antagonism of the D₂-like receptors (Seeman et al., Proc. Nat. Acad. Sci. USA 72:4376-4380, 1975), and antagonism of the D₄ receptor subtype provides a better correlation than do the D₂ or D₃ subtypes, implicating the D₄ receptor as the most likely target of neuroleptic drugs (Seeman and Van Tol, Trends Pharmacol Sci. 15:264-270, 1994).

Generally, a receptor must be occupied by its agonist or partial agonist in order to attain its active conformation and convey the neurotransmitter signal. However, in a phenomenon known as "spontaneous receptor activity," it is possible for a receptor to maintain the active conformation even in the absence of agonist occupation, although the extent of this phenomenon appears to be dependent upon prior exposure of the cells to the appropriate agonist.

Without wishing to be bound by theory, the binding of the agonist apparently induces a conformational change in the receptor, causing it to become active. In this active state, a modifiable amino acid residue (a "hot spot") on the intracellular portion of the receptor, exactly 18 residues (5 helical turns) from the agonist binding site, becomes accessible to a native enzyme. This enzyme modifies the "hot spot" such that the modification (typically a phosphorylation in other spontaneously active receptors but adenosylation in the D₄ receptor) prevents the receptor from returning to the inactive conformation upon departure of the agonist. In

fact, the receptor remains in the active conformation and continues to propagate the neurotransmitter signal, until the modification is removed by subsequent enzyme activity.

5 The dopamine D₄ receptor, methionine #313 (human D₄ receptor numbering) has been identified as such a "hot spot"; that is, referring to Fig. 1, methionine #313 is a modifiable amino acid residue on the intracellular portion of the D₄ receptor, located 18 residues (exactly 5 helical turns) below the key residue which is utilized by agonists to induce the active receptor conformation (Deth, "Compositions and Methods for Detecting Schizophrenia," WO 96/37780).

15 It has been shown that any modification of residues occupying the same position as dopamine D₄ receptor methionine #313 in other receptors will cause the receptor to become spontaneously active and exhibit spontaneous activity (Samama et al., J. Bio. Chem. 268:4625-4636, 1993). Similarly, it is believed that the binding of dopamine to the D₄ receptor causes an alteration of the receptor conformation to the active conformation. As a result of this alteration, 20 methionine #313 becomes accessible to MAT, and MAT adds an adenosyl group to the sulfur atom of the methionine. This modification of methionine #313 by MAT prevents the re-configuration of the receptor to the inactive form upon departure of the agonist. Thus, the dopamine D₄ receptor is 25 capable of spontaneously maintaining its active conformation, i.e., maintaining its active conformation without dopamine occupation. This active conformation is maintained until the modification (e.g., the adenosyl group) is removed by subsequent enzyme activity.

30 While phosphorylated threonine residues can be restored to their native state by the action of phosphatase enzymes, the fate of S-adenosylated methionine residue #313 was not immediately obvious. However, by analogy to S-adenosylmethionine, it was considered that the terminal 35 methyl group may be available for donation in a

methytransferase reaction. The location of the "hot spot" residue is at the cytoplasmic surface of the plasma membrane, and it is thus located at or near the head groups of membrane phospholipids, raising the possibility that the S-adenosylated form of the D₄ receptor might serve as a donor of methyl groups for methylation of the phospholipid phosphatidylethanolamine (PE) in the stepwise synthesis of phosphatidylcholine (PC).

Phosphatidylethanolamine (PE) is sequentially N-methylated by the action of two enzymes, phospholipid methyltransferases I and II (PLMT I and II), located on the inner and outer sides of the plasma membrane respectively (Hirata et al., Science 209:1082, 1980). Formation of N-monomethyl PE by PLMT I has been linked to the control of the membrane microviscosity, with increased formation leading to decreased microviscosity (i.e., increased membrane fluidity).

To determine whether D₄ receptors might play a role in phospholipid methylation, CHO cells transfected with human D₄ receptor were incubated with [³H]-methyl-methionine for 1 hr. in the presence or absence of dopaminergic ligands and GTP, followed by extraction of phospholipids and determination of [³H] incorporation. The basal level of phospholipid methylation, however, could be augmented by about 30% by addition of a combination of GTP(1 mM) and dopamine (10 µM).

The inclusion of the dopamine antagonists haloperidol (1 µM) (or clozapine) produced a decrease in phospholipid methylation to a level which was approximately 75% below the original basal level. In studies with PC12 cells, haloperidol treatment caused a similar decrease in phospholipid methylation, suggesting that dopamine receptor-dependent phospholipid methylation may be a feature of a number of cell types, including neuronal tissues.

An impairment in methylation reactions has previously been suggested to accompany and to cause schizophrenia. In an early hypothesis ("the transmethylation hypothesis"), a

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classical methionine cycle provides for methyl donation to phospholipid methyltransferase I (PLMT I) by formation of S-adenosylmethionine via the action of methionine adenosyltransferase (MAT). After methyl transfer, 5 adenosylhomocysteine hydrolase (Ado Hcyase) removes the adenosyl moiety from adenosylhomocysteine and methionine is reformed via the action of methionine synthase (Met Synthase) to complete the cycle. Methionine synthase utilizes cobalamin (Vit B₁₂) to effect the methylation of homocysteine, 10 with 5-methyl tetrahydrofolate (5-methyl THF) serving as the primary methyl donor. Serine hydroxymethyltransferase (Serine HMT) forms 5,10-methylene THF from the conversion of serine to glycine. The active R* form of the dopamine D₄ receptor (D₄^{*MET}) is adenosylated at M313 by MAT to yield D₄^{*SAM} 15 which can serve as a donor of methyl groups for N-methylation of PE by PLMT I. The remainder of the D₄ cycle is hypothesized to involve the actions of AdoHcyase and Met Synthase. Binding of negative antagonists to the inactive D₄ state of the receptor can serve to reduce its participation 20 in phospholipid methylation. As a transmembrane protein, the D₄ receptor may be conformationally sensitive to changes in membrane fluidity, creating the potential for negative feedback regulation.

Clearly the net amount of PE methylation will depend 25 upon three factors: 1) the concentration of the substrate PE; 2) availability of the methyl donors SAM and D₄^{*SAM}; and 3) the catalytic activity provided by PLMT I. Availability of D₄^{*SAM} is dependent upon a number of factors including the density of D₄ receptors and the prevailing level of the D₄^{*MET} 30 state. Both SAM and D₄^{*SAM} are dependent upon MAT activity and the efficiency of those events which allow for restoration of the methionine form of the receptor after methyl donation, some of which are outlined in Fig. 2. In short, the dynamics of the methionine cycle of methyl donation determine the 35 ability of both SAM and the D₄ receptor to supply methyl

groups for phospholipid methylation.

The following examples are presented to illustrate the advantages of the present invention and to assist one of ordinary skill in making and using the same. These examples are not intended in any way otherwise to limit the scope of the disclosure.

EXAMPLE I

Involvement of a D₄ dopamine receptor
in phospholipid methylation.

Incorporation of [³H] label into phospholipids was measured in intact CHO cells transfected with the D₄ receptor gene (CHO/D₄ cells) after incubation with [³H-methyl] methionine. The media of cells growing in monolayer was changed to 1 ml of Hank's balanced salt solution, nucleoside free, containing 8 µCi/ml [³H-methyl] methionine. After a 60 min incubation, 1.5 ml of ice-cold 10% trichloroacetic acid (TCA) was added, and cells were harvested and centrifuged. The pellet was washed with 2.5 ml of TCA and extracted with 15 ml of a 6:3:1 mixture of CHCl₃/CH₃OH/HCl (2 M). The CHCl₃ phase was washed twice with 4 ml of 0.1 M KCl in 50% CH₃OH, and an aliquot of the phospholipid-containing CHCl₃ was dried and counted. The graph depicted in Fig. 3a shows cells treated with either vehicle (Control), the D₄ receptor-selective agonists GTP (1 mM) and dopamine (10 µM) (DA/GTP) or both GTP and dopamine plus the D₄ receptor-selective antagonist haloperidol (1 µM) (HALO) at the start of [³H-methyl]methionine incubation. A small stimulation of phospholipid methylation by the agonists GTP and dopamine and a large inhibition when the antagonist haloperidol is simultaneously included was observed. Fig. 3b shows dose-dependent inhibition of [¹⁴C] formate-labelled phospholipid methylation in CHO/D₄ cells grown in nucleoside-free medium by the highly D₄-selective antagonist U-101,958. Fig. 3c shows dose-dependent dopamine stimulation of [¹⁴C] formate-

labelled phospholipid methylation in SK-N-MC cells, which are derived from a human neuroblastoma and naturally contain the D₄ receptor, grown in the presence of nucleosides.

Referring again to Fig. 3b, dose-response studies in CHO cells with the D₄ receptor-selective antagonist U-101,958 conducted in the absence of agonist indicated that its inhibitory effects occurred at concentrations consistent with occupation of dopamine D₄ receptors, with an IC₅₀ of 0.2 nM as compared to its reported K_b of 1 nM (Schlacter et al., Soc. Neurosci. Abstr. 21:252.7, 1995). Similar results were obtained with another D₄ selective antagonist L-745,870. This inhibition verifies that spontaneous activity of the D₄ receptor does indeed play an important role in phospholipid methylation, especially under nucleoside free growth conditions. The magnitude of antagonist effects suggest that the D₄ receptor can serve as a significant source of methyl groups, supplemented by a dopamine antagonist-insensitive component which likely represents the contribution of S-adenosyl methionine itself.

EXAMPLE II

Phospholipid methylation in lymphocytes
from persons with schizophrenia vs. normal controls

To determine whether D₄ receptor-dependent phospholipid methylation was altered in schizophrenia, the labelling of phospholipids by [³H]-methyl-methionine in lymphocytes obtained from people with schizophrenia (as defined by DSM IV diagnostic criteria Diagnostic and Statistical Manual of Mental Disorders (4th Edition), American Psychiatric Association, Washington, D.C., 1987, pp. 759-764) under medical treatment) was measured as compared to lymphocytes from healthy controls. Lymphocytes were isolated as previously described (De La Rosa et al., J. Biol. Chem. 267:10699, 1992) and aliquots of 4 X 10⁶ cells were resuspended in Hank's balanced salt solution containing 8 µCi/ml [³H-methyl]methionine for 60 min. Dopamine (10 µM)

and GTP (1 mM) with or without haloperidol (1 μ M) were added to additional groups. Incorporation of [³H-methyl] label into phospholipids was determined as described in Example I.

(A) Individual basal levels of phospholipid methylation and their mean \pm SEM. (B) Influence of dopamine/GTP and haloperidol on phospholipid methylation in control and schizophrenia samples. Differences between control and patient values are significant ($p < 0.001$) for all treatment groups. The mean basal level of phospholipid methylation in patient samples was less than one-third the value of controls, indicating a major deficit in this process (Fig. 4A). In both groups, the addition of dopamine and GTP caused a significant increase in methyl incorporation while haloperidol caused a significant decrease (Fig. 4B), but the percentage changes in control were less than had been observed in CHO cells. However, these effects were relatively exaggerated in patient lymphocytes, associated with their low initial values. The difference between the agonist-stimulated and antagonist-inhibited values, which can be considered as the potential contribution dopamine receptors can make to this process, was only 20% smaller in the schizophrenia group. Thus while D₄ dopamine receptors are important in determining the level of phospholipid methylation in isolated lymphocytes, especially in individuals with schizophrenia, the large deficiency in schizophrenia appears to involve aspects other than the receptor contribution *per se*.

MATERIALS AND METHODS

Materials. U-101,958 maleate and L-745,870 hydrochloride, two examples of D₄ receptor-selective antagonists, were purchased from Research Biochemicals International (Natick, MA). [¹⁴C] - formic acid was obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO).

Cell Preparations. CHO cells transfected with the D₄ receptor gene were fed with minimum essential medium (alpha modifications, with or without nucleosides) (pH 7.2) containing 2mM L-glutamine, W/G418 antibiotic (400 mg/L), donor horse serum (2.5%), fetal bovine serum (2.5%), penicillin/streptomycin/fungizone (1%), CuSO₄ (10⁻⁶M). Cells (200,000 cells/well) were plated in 6-well- plates and placed in 5% CO₂ incubator at 37°C.

Phospholipid Methylation Assay. Two-day-old confluent, cultured D₄-transfected CHO cells were used for assay. The pH of Hank's balanced buffer (HBSS) was adjusted to pH 7.4 using sodium bicarbonate buffer. [¹⁴C]-formic acid (1 µCi/ml) and pharmacological agents were added to HBSS. Feeding medium was removed from the well and then 600 µl of the HBSS solution with the radiolabelled formic acid (warmed to 37°C) was added to each well of 6-well-plates. Plates were incubated at 37°C for 30 minutes in the culture incubator. The reaction was stopped by washing once with ice-cold PBS (pH 7.4) and, after aspirating off the HBSS, 500 µl of 10% trichloroacetic acid (TCA) was added. Cells were harvested by scraping with a cell scraper and transferred into a plastic microcentrifuge tube. Another 500 µl of TCA was added to rinse and transferred into the same tube. A sample (100 µl) was taken out for Lowry protein assay after the cells were homogenized. Each tube was spun in a bench top minifuge at 12,000 rpm for 15 minutes. After the TCA supernatant was aspirated off, 2N HCl (150 µl) and 100% methanol (450 µl) were added to each tube. The pellets were homogenized and 900 µl of chloroform (CHCl₃) was added to each tube. The sample tubes were placed on a shaker for 1 hour at room temperature to allow for phase separation and the top aqueous layer was removed by aspiration after shaking. 0.1 M KCl in 50% methanol (500 µl) was then added

and the top aqueous layer was carefully removed by aspiration after thorough vortexing. The CHCl_3 layer was saved for scintillation counting. 300 μl of the CHCl_3 layer was transferred to a counting vial and evaporated to dryness by a heated water bath. Six replicate values were obtained for each experimental group. CPM values were normalized to the protein content of each sample and expressed as the mean \pm S.E.M. for the group. Differences between groups were analyzed by a t-test with $p < 0.05$ as the criterion for significance.

USE

The metabolic relationships described herein have been used to develop methods of identifying therapeutic processes or agents for treating schizophrenia or a related neuropsychiatric disorder. These screening methods include establishing a cultured cell line, either naturally expressing D_4 receptor or transfected with the D_4 receptor gene, as an assay system; making an initial determination of the level of phospholipid methylation in the cultured cells; administering the candidate therapeutic process or agent to be assessed to the cultured cell assay system; and determining the level of phospholipid methylation following introduction of the candidate process or agent. An increase in the level of phospholipid methylation subsequent to administration of the candidate therapeutic process or agent indicates that the candidate process or agent is potentially therapeutically effective for treating schizophrenia or a related neuropsychiatric disorder. The particular influence of the candidate therapeutic process or agent on D_4 receptor-dependent phospholipid methylation is assessed by a comparison of agonist stimulated VS. antagonist-inhibited methylation levels. An increase in the receptor component indicates a potentially useful effect on dopamine function in the brain.

A similar method can be used to assess the effectiveness of the therapeutic process or agent for treating schizophrenia or a related neuropsychiatric disorder in a patient.

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While the present invention has been described in conjunction with a preferred embodiment, one of ordinary skill, after reading the foregoing specification, will be able to effect various changes, substitutions of equivalents, and other alterations to the compositions and methods set forth herein. It is therefore intended that the protection granted by Letters Patent hereon be limited only by definitions contained in the appended claims and equivalents thereof.

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